

Long-term Effect of Convulsive Behavior on the Density of Adenosine A₁ and A_{2A} Receptors in the Rat Cerebral Cortex

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Summary: *Purpose:* Adenosine is a neuromodulator that has been proposed to act as an anticonvulsant mainly via inhibitory A₁ receptors, but recent data show that genetic deletion of facilitatory A_{2A} receptors might also attenuate convulsions. Since both A₁ and A_{2A} receptors are prone to down- and upregulation in different stressful situations, we investigated if convulsive behavior leads to a long-term change in A₁ and A_{2A} receptor density in the rat cerebral cortex.

Methods: Stage 4–5 convulsions (Racine's scale) were induced in adult Wistar rats either through amygdala stimulation (kindling) or by intraperitoneal injection of kainate (10 mg/ml). Rats were killed after 4 weeks to evaluate adenosine A₁ and A_{2A} receptor density in the cerebral cortex using both Western blot and membrane binding assays.

Results: The binding density of the A₁ antagonist, ³H-DPCPX, decreased by 40. ± 4.4% and by 20.7 ± 0.5% after kindling or kainate injection. Likewise, A₁ receptor immunoreactivity in cortical membranes from kindled or kainate-injected rats decreased by 19.1 ± 3.3% and 12.7 ± 5.7%, respectively. In contrast, the binding density of the A_{2A} receptor antagonist ³H-SCH 58261 increased by 293 ± 34% and by 159 ± 32% in cortical membranes from kindled or kainate-injected rats, and A_{2A} receptor immunoreactivity also increased by 151 ± 12% and 79.6 ± 7.0%.

Conclusions: This indicates that after convulsive behavior there is a long-term decrease of A₁ receptors accompanied by an increased density of A_{2A} receptors, suggesting that A_{2A} antagonists rather than A₁ agonists may be more promising anticonvulsive drugs. **Key Words:** Adenosine—Cortex—Kindling—Kainate—Epilepsy—A₁ receptor—A_{2A} receptor.

Adenosine is a ubiquitous neuromodulator that mainly inhibits synaptic transmission and neuronal excitability through activation of the predominant adenosine A₁ receptors (1). The ability of adenosine to selectively depress glutamatergic excitatory pathways makes A₁ receptors interesting anticonvulsant targets [reviewed in (2)]. Accordingly, exogenous administration of A₁ receptor agonists attenuates seizures, and the use of A₁ receptor antagonists has proconvulsant effects [reviewed in (2)]. It has even been proposed that the loss of the A₁ receptor-mediated control of glutamatergic function could contribute to the implementation of epileptic status (3). However, conflicting results have been reported in relation to the impact of convulsive behavior on cortical A₁ receptors [reviewed in (4)]. In other brain regions, chronic stressful situations, such as amygdala kindling (5) or Alzheimer's disease (6), decreased the density of A₁ receptors.

Adenosine can also activate another less abundant adenosine receptor subtype, i.e., A_{2A} receptors, with effects generally opposite to these mediated by A₁ receptors (7). These facilitatory A_{2A} receptors are most abundant in the basal ganglia and have a density 20 times lower in the cerebral cortex (8). However, despite their low abundance, and by mechanisms still to be resolved, pharmacological blockade or genetic inactivation of these A_{2A} receptors confer robust neuroprotection in the limbic and neocortex in different noxious brain situations [reviewed in (9)]. In particular, genetic inactivation of A_{2A} receptors decreases ethanol withdrawal-induced seizures (10), suggesting a role for A_{2A} receptors also in the control of convulsive behavior. Interestingly, studies in nonbrain preparations have documented that prolonged stressful situations, such as hypoxia (11) or exposure to cytokines (12), enhance the expression and density of these A_{2A} receptors, but this has not yet been documented in brain tissue.

Therefore, there is ground to consider both A₁ and A_{2A} receptors as possible targets for the development of anticonvulsants. However, since noxious situations lead to

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changes in the density of both A₁ and A_{2A} receptors, it appears necessary to determine if the occurrence of seizures might also lead to changes in A₁ and A_{2A} receptor densities. Thus, this study was designed to investigate the long-term changes of the densities of A₁ and A_{2A} receptors in the cerebral cortex using two different strategies to induce episodic convulsive behavior: amygdala kindling (13) and intraperitoneal injection of kainate (14).

METHODS

Reagents

³H-1,3-Dipropyl-8-cyclopentyladenosine (³H-DPCPX, specific activity 109.0 Ci/mmol) was from DuPont NEN (Anagene, Portugal), and ³H-5-amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine (³H-SCH 58261, specific activity 77 Ci/mmol) was prepared by Amersham (Buckinghamshire, U.K.) and was a generous gift of Dr. Ennio Ongini (Shering-Plough, Milan, Italy). Kainate and adenosine deaminase (calf intestine suspension, 2000 U/ml, EC 3.5.4.4) were from Sigma (Reagente 5, Oporto, Portugal), 8-{4-[(2-aminoethyl)amino]carbonylmethyl-oxyphenyl}xanthine (XAC) was from Research Biochemical International (Reagente 5, Oporto, Portugal), goat purified IgG anti-adenosine A_{2A} receptor antibody (200 µg/ml) was from Santa Cruz Biotechnology-Europe (Freelab, Lisbon, Portugal), and rabbit purified IgG antiadenosine A₁ receptor antibody (1.8 mg/ml) was from Affinity Bioreagents (Golden City, CA, U.S.A.).

Animals and convulsive models

The experiments were performed using male Wistar rats from Harlan Ibérica (Barcelona, Spain) weighing 240–280 g for the amygdala-kindling experiments and 160–180 g for the kainate-injection experiments. The rats were handled according to the EU guidelines for use of experimental animals (86/609/EEC); they were anesthetized under halothane atmosphere before being killed by decapitation.

For the amygdala-kindling studies, the rats were divided into three groups: control rats, fully kindled rats, and sham-operated rats (electrode implemented but no stimulation delivered). Kindling was achieved by insertion of an electrode in the amygdala to allow chronic sub-threshold stimulation to implement a fully kindled state, as previously described (5). Briefly, the rats were anesthetized with sodium pentobarbitone 60 mg/kg (3% wt/vol in saline) and coaxial bipolar electrodes (David Kopf) were implanted to the left amygdala. After a postoperative recovery of 1 week, animals were stimulated twice a day (10 a.m. and 4 p.m.) with a 50 Hz, 1 s, 500 µA, square wave with a positive pulse of 1 ms (Hugo Sachs stimulator, March-Hugstetten, Germany). After five consecutive stage 5 convulsions, fully kindled rats were allowed a 4-week recovery period before being killed for in vitro studies.

For the kainate injection studies, the rats were divided into two groups: control (saline-injected) and kainate-injected animals. Kainate (10 mg/ml) was injected intraperitoneally and the animal was placed in an observation box facing two experimenters who recorded the behavior changes displayed by the animals during a period of 4 hours according to the Racine's scale. Kainate-injected animals displayed a convulsive behavior that ranked at stage 4 (two rats) and stage 5 (one rat), whereas two other rats also reached stage 5 but died within the next hour, thus being excluded from the study. The rats were then allowed a 4-week recovery period before being killed for in vitro studies.

Adenosine receptor binding studies in cortical membranes

Saturation binding curves of the selective A₁ receptor antagonist, ³H-DPCPX, or of the selective A_{2A} receptor antagonist, ³H-SCH 58261, were performed as previously described using whole membranes from the cerebral cortex (8,15). Binding of ³H-DPCPX (0–10 nM) or of ³H-SCH 58261 (0–10 nM) was for 2 or 1 hours, respectively, at 37°C, with 32–159 µg of membrane protein in a final volume of 200 µl in an incubation solution containing 50 mM Tris-HCl and 10 mM MgCl₂, pH 7.4, with 2 U/ml adenosine deaminase. The binding reactions were stopped by vacuum filtration through Whatman GF/C glass fiber filters, followed by washing of the filters and reaction tubes with 8 ml of the incubation solution, kept at 4°C. Radioactivity retained in the filters was then determined after addition of 4 ml of scintillation liquid (Scintran Cocktail T, Wallac, Turku, Finland). Results are expressed as specific binding, determined by subtraction of the nonspecific binding, which was measured in the presence of 2 µM XAC, and normalized per amount of protein. All binding assays were performed in duplicate. To derive the binding parameters from saturation curves (K_D and B_{max} values), the data were fitted by a rectangular hyperbola using the GraphPad Prism software (San Diego, CA, U.S.A.).

Western blot analysis of adenosine receptor immunoreactivity in cortical membranes

Adenosine A₁ and A_{2A} receptor immunoreactivity was evaluated by Western blot analysis, as previously described (5,15) in whole membranes from the rat cerebral cortex. Cortical membranes solubilized in 5% SDS were separated by SDS-PAGE (13%) and electrotransferred to polyvinylidene difluoride (PVDF) membranes (0.45 µm from Amersham). After blocking, the membranes were incubated overnight at 4°C with either the antiadenosine A₁ receptor (1:1000 dilution) or anti-A_{2A} receptor antibodies (1:500 dilution), then with the alkaline phosphatase-conjugated anti-rabbit or anti-goat secondary antibody (1:10,000 dilution from Amersham Little Chalfont, Buckinghamshire, U.K.), and finally with Enhanced Chemi-Fluorescence (Amersham) for 5 min, then

analyzed densitometrically with Quantity one, Versadoc 3000 (BioRad).

Statistics

The values presented are mean \pm SEM of *n* experiments. Comparison between the different animal groups was made using the two-tailed Mann–Whitney test. Statistical significance was considered at $p < 0.05$.

RESULTS

Modification of cortical A₁ and A_{2A} receptors in kindled rats

The selective A₁ receptor antagonist, ³H-DPCPX, bound with a K_D of 0.77 nM (95% confidence interval: 0.58–0.96 nM, *n* = 4) and a B_{max} of 917 \pm 60 fmol/mg protein (*n* = 4) to whole membranes from the cerebral cortex of control rats. As illustrated in Fig. 1A, there was a decrease of ³H-DPCPX binding to cortical membranes obtained from kindled rats. In fact, in cortical membranes from kindled rats, the B_{max} of ³H-DPCPX binding was decreased (40.4 \pm 4.4%, $p < 0.05$) to 546 \pm 52 fmol/mg

protein (*n* = 4), whereas the K_D of ³H-DPCPX binding was not significantly ($p > 0.05$) modified (K_D = 0.81 nM, 95% confidence interval of 0.45–1.37 nM, *n* = 4). This decreased density of A₁ receptors was not due to the surgical procedure since the K_D (0.77 nM, 95% confidence interval of 0.43–1.11 nM, *n* = 4) and the B_{max} of ³H-DPCPX binding (866 \pm 23 fmol/mg protein, *n* = 4) in cortical membranes from sham-operated rats were not different ($p > 0.05$) from control rats (Fig. 1A).

To confirm that there was indeed a decreased density of A₁ receptors in cerebral cortical membranes of kindled compared to control rats, we compared by Western blot analysis the immunoreactivity against A₁ receptors in membranes from cortical membranes of control and fully kindled rats. As illustrated in Fig. 1B, there was a decrease in the densitometrically measured anti-A₁ receptor immunoreactivity in cortical membranes from kindled compared to control rats, with an average value of 19.1 \pm 3.3% (*n* = 3).

The selective A_{2A} receptor antagonist, ³H-SCH 58261, bound with a K_D of 0.98 nM (95% confidence interval: 0.47–1.49 nM, *n* = 4) and a B_{max} of 27.7 \pm 3.1 fmol/mg protein (*n* = 4) to whole membranes from the cerebral cortex of control rats. As illustrated in Fig. 2A, there was a marked increase of ³H-SCH 58261 binding to cortical membranes obtained from kindled rats. In fact, in cortical membranes from kindled rats, the B_{max} of ³H-SCH 58261 binding was nearly tripled (293 \pm 34% increase, $p < 0.05$) to 109.0 \pm 3.5 fmol/mg protein (*n* = 4), with no change ($p > 0.05$) of K_D (1.07 nM, 95% confidence interval of 0.65–1.50 nM, *n* = 4). This increased density of A_{2A} receptors was not due to the surgical procedure since the K_D (0.88 nM, 95% confidence interval of 0.41–1.36 nM, *n* = 4) and the B_{max} of ³H-SCH 58261 binding (30.6 \pm 3.4 fmol/mg protein, *n* = 4) in cortical membranes from sham-operated rats were not different ($p > 0.05$) from control rats (Fig. 2A).

To confirm that there was indeed an increased density of A_{2A} receptors in cerebral cortical membranes of kindled compared to control rats, we compared by Western blot analysis the immunoreactivity against A_{2A} receptors in membranes from cortical membranes from control and fully kindled rats. As illustrated in Fig. 2B, there was an increase in the densitometrically measured anti-A_{2A} receptor immunoreactivity in cortical membranes on kindled compared to control rats, with an average value of 151 \pm 12% (*n* = 3).

Modification of cortical A₁ and A_{2A} receptors in kainate-injected rats

To further strength the idea that a convulsive period caused a long-term effect on the density of adenosine receptors in the cerebral cortex, we decided to use a different experimental paradigm to trigger convulsions. Thus, rats were injected with kainate, which induced convulsions

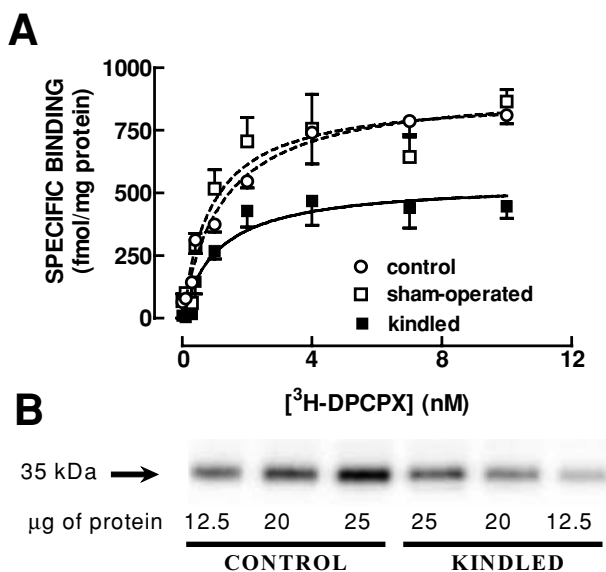


FIG. 1. Long-term effect of convulsions induced by amygdala kindling on the density of adenosine A₁ receptors in the rat cerebral cortex. **A:** Average saturation binding curves with the selective A₁ receptor antagonist ³H-DPCPX in cerebral cortical membranes of control (open circles), sham-operated (open rectangles), and fully kindled rats (filled rectangles). The ordinates represent the specific binding of ³H-DPCPX, obtained on subtraction of the nonspecific binding, determined in the presence of 2 μ M XAC from total binding. The results are mean \pm SEM of four experiments. **B:** A Western blot comparing the A₁ receptor immunoreactivity in membranes from the rat cerebral cortex of control (first three lanes from the left) and fully kindled rats (first three lanes from the right). The SDS-PAGE gel was loaded with three different amounts of cortical protein from each group of rats and is representative of three similar separations carried out with membranes prepared from different animals. It is evident that the intensity of bands identified as the A₁ receptor protein is systematically lower in the membranes derived from kindled rats when compared to the same amount of loaded cortical membrane from control rats.

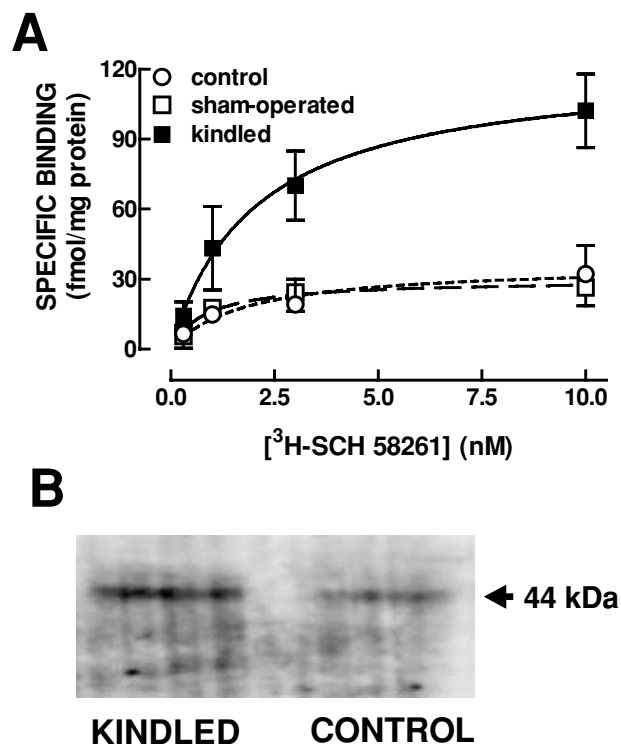


FIG. 2. Long-term effect of convulsions induced by amygdala kindling on the density of adenosine A_{2A} receptors in the rat cerebral cortex. **A:** Average saturation binding curves with the selective A_{2A} receptor antagonist ^3H -SCH 58261 in cerebral cortical membranes of control (open circles), sham-operated (open rectangles), and fully kindled rats (filled rectangles). The ordinates represent the specific binding of ^3H -SCH 58261, obtained on subtraction of the nonspecific binding, determined in the presence of $2\ \mu\text{M}$ XAC from total binding. The results are mean \pm SEM of four experiments. **B:** A Western blot comparing the A_{2A} receptor immunoreactivity in membranes from the rat cerebral cortex of control (right lane) and fully kindled rats (left lane). The SDS-PAGE gel was loaded with $200\ \mu\text{g}$ of cortical protein from each group of rats and is representative of three similar separations carried out with membranes prepared from different animals. It is evident that the intensity of bands identified as the A_{2A} receptor protein is higher in the membranes derived from kindled compared to control rats.

reaching a stage 4-5 between 30 and 240 min after kainate injection, and the density of cortical A_1 and A_{2A} receptors was evaluated after 30 days. As was observed in amygdala-kindled rats, there was a decrease in the binding density of the selective A_1 receptor antagonist, ^3H -DPCPX. Thus, ^3H -DPCPX bound with a K_D of $1.04\ \text{nM}$ (95% confidence interval: 0.50 – $1.51\ \text{nM}$, $n = 3$) and a B_{max} of $907 \pm 4.5\ \text{fmol/mg protein}$ ($n = 3$) to whole membranes from the cerebral cortex of control (i.e., saline-injected rats). As illustrated in Fig. 3A, in cortical membranes from kindled rats, the B_{max} of ^3H -DPCPX binding was decreased to $719 \pm 15\ \text{fmol/mg protein}$ ($20.7 \pm 0.5\%$ decrease, $p < 0.05$, $n = 3$) without change in K_D ($K_D = 1.03\ \text{nM}$, 95% confidence interval of 0.82 – $1.24\ \text{nM}$, $n = 3$). This decreased density of A_1 receptors was also confirmed by Western blot analysis. In fact, as illustrated in Fig. 3B,

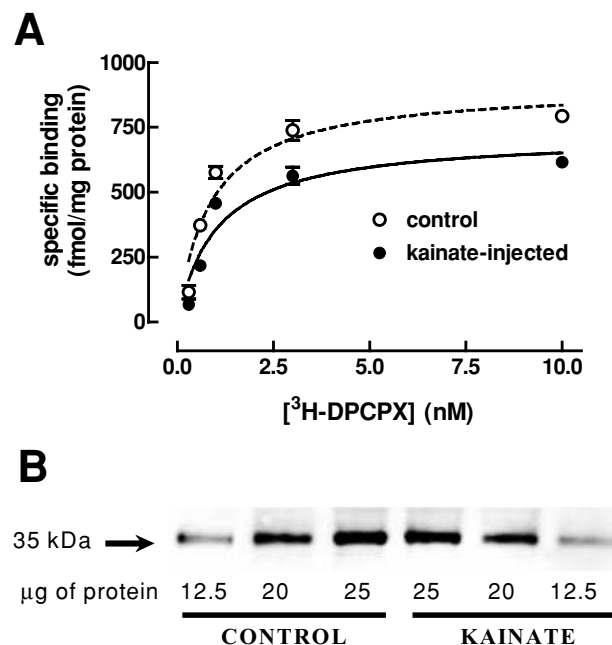


FIG. 3. Long-term effect of convulsions caused by the intraperitoneal injection of kainate on the density of adenosine A_1 receptors in the rat cerebral cortex. **A:** Average saturation binding curves with the selective A_1 receptor antagonist ^3H -DPCPX in cerebral cortical membranes of control (open circles) and kainate-induced kindled rats (filled rectangles). The ordinates represent the specific binding of ^3H -DPCPX, obtained on subtraction of the nonspecific binding, determined in the presence of $2\ \mu\text{M}$ XAC from total binding. The results are mean \pm SEM of three experiments. **B:** A Western blot comparing the A_1 receptor immunoreactivity in membranes from the rat cerebral cortex of control (first three lanes from the left) and fully kindled rats (first three lanes from the right). The SDS-PAGE gel was loaded with three different amounts of cortical protein from each group of rats and is representative of three similar separations carried out with membranes prepared from different animals. It is evident that the intensity of bands identified as the A_1 receptor protein is systematically lower in the membranes derived from kainate-injected rats when compared to the same amount of loaded cortical membrane from control rats.

there was a decrease in the densitometrically measured anti- A_1 receptor immunoreactivity in cortical membranes of kainate-injected compared to control rats, with an average value of $12.7 \pm 5.7\%$ ($n = 3$).

Convulsions induced by kainate also caused a marked upregulation of A_{2A} receptors, as occurred in kindled rats. In fact, as illustrated in Fig. 4, there was a near doubling ($159 \pm 32\%$ increase, $p < 0.05$) of the density of ^3H -SCH 58261 binding in kainate-injected rats (B_{max} of $70.2 \pm 5.8\ \text{fmol/mg protein}$, $n = 3$) compared to control (B_{max} of $26 \pm 2.9\ \text{fmol/mg protein}$, $n = 3$), with no change in K_D ($0.89\ \text{nM}$, 95% confidence interval: 0.78 – $1.01\ \text{nM}$, $n = 3$, in control vs. $0.91\ \text{nM}$, 95% confidence interval: 0.59 – $1.21\ \text{nM}$, $n = 3$, in kainate-injected rats). This increased density of A_{2A} receptors was also confirmed by Western blot analysis. In fact, as illustrated in Fig. 4B, there was a marked increase in the densitometrically measured anti- A_{2A} receptor immunoreactivity in cortical membranes of

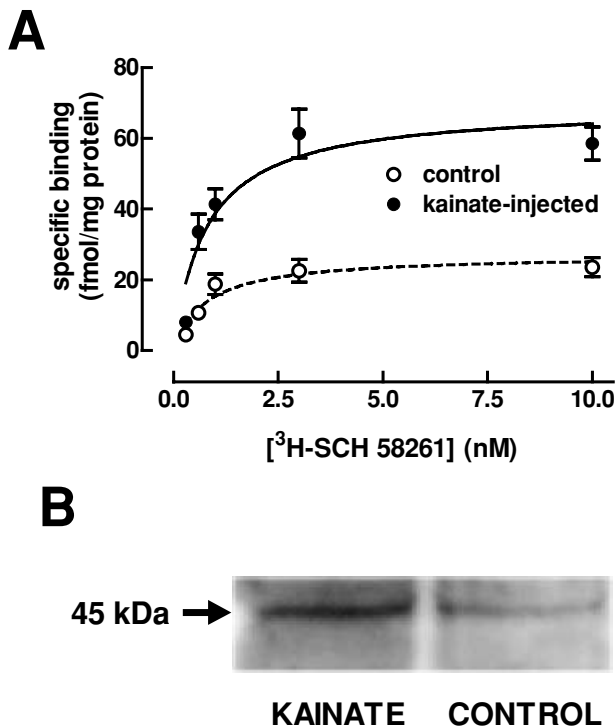


FIG. 4. Long-term effect of convulsions caused by the intraperitoneal injection of kainate on the density of adenosine A_{2A} receptors in the rat cerebral cortex. **A:** Average saturation binding curves with the selective A_{2A} receptor antagonist ³H-SCH 58261 in cerebral cortical membranes of control (open circles) and kainate-induced kindled rats (filled rectangles). The ordinates represent the specific binding of ³H-SCH 58261, obtained on subtraction of the nonspecific binding, determined in the presence of 2 μ M XAC from total binding. The results are mean \pm SEM of three experiments. **B:** A Western blot comparing the A_{2A} receptor immunoreactivity in membranes from the rat cerebral cortex of control (right lane) and kainate-injected rats (left lane). The SDS-PAGE gel was loaded with 180 μ g of cortical protein from each group of rats and is representative of three similar separations carried out with membranes prepared from different animals. It is evident that the intensity of bands identified as the A_{2A} receptor protein is higher in the membranes derived from kindled compared to control rats.

kainate-injected compared to control rats, with an average value of $79.6 \pm 7.0\%$ ($n = 3$).

DISCUSSION

The neuromodulator adenosine has been associated with epileptic phenomena [reviewed in (2)] since there is a massive amount of adenosine released during seizures (16) and manipulation of the activity of either A₁ [reviewed in (2)] or A_{2A} receptors (10) affects convulsive behavior. We now found that convulsive behavior triggers a long-term decrease of A₁ receptor density and an increase of A_{2A} receptor density in the cerebral cortex. This conclusion was reached using two different and well-established paradigms to trigger convulsive behavior, amygdala kindling (13) and kainate injection (14), and two different experimental approaches to measure the

density of adenosine A₁ and A_{2A} receptors, i.e., radioligand binding with selective antagonists and using selective antibodies against each adenosine receptor subtype. Thus, the observed remarkable qualitative similarity between the findings obtained when using two different ways to trigger convulsions and two different methods to evaluate changes in adenosine receptor density emphasizes the robustness of this major conclusion, i.e., that convulsive behavior causes a long-term increase in A_{2A} receptor density and a decrease of the density of A₁ receptors.

Several studies have shown that A₁ receptors undergo desensitization on prolonged activation (17–19), which is expected due to the massive and prolonged increase of extracellular adenosine occurring upon ictal activity (16,20,21). However, previous studies failed to reach an agreement on the effects of seizure activity on the density of A₁ receptors in the brain [reviewed in (4)]. In fact, most studies evaluating acute or short-term effects (i.e., after 24 h) of convulsions most commonly found an increase in the density of A₁ receptors in different brain regions, either upon induction of seizures with pentylenetetrazole (22,23), bicuculline (24,25), or 3-mercaptopropionate (26). In contrast, no acute or short-term changes in the density of brain A₁ receptors were found after electroconvulsive stimulation (27,28) or upon intraperitoneal injection of kainate (29). Changes in the density of A₁ receptors occurring 2–15 days after the observation of convulsive behavior also seem to depend on the strategy used to trigger convulsions. Thus, convulsions triggered by pentylenetetrazole (22) or repeated electroconvulsive stimulation (27,28) enhanced the density of A₁ receptors in most brain regions, whereas no measurable modification of A₁ receptor density was reported upon amygdala kindling (30) or kainate injection (29,31). Interestingly, most studies seem to find a long-term decrease in the density of adenosine A₁ receptors, at least in the hippocampus (5,29,31). Thus, there is a general trend indicating that convulsive behavior leads to a long-term decrease in the density of A₁ receptors, as we now report has occurred in the cerebral cortex. This conclusion is in general agreement with the development of tolerance in relation to the anticonvulsive effects of A₁ receptor agonists (3,32).

However, the currently observed decrease of A₁ receptor density does not necessarily exclude A₁ receptors as potential targets for the development of new anticonvulsant drugs. In fact, while the efficiency of most anticonvulsants decreases markedly with increasing severity of seizures, A₁ receptor activation is able to suppress seizures in an animal model of pharmacoresistant epilepsy (33). This may be understood if one keeps in mind that seizures cause a long-term greater modification of the extracellular levels of adenosine able to activate A₁ receptors than a desensitization of responses mediated by A₁ receptors [(5), reviewed in (4)]. This suggests that strategies aimed at increasing the extracellular levels of adenosine may be more

effective than using A₁ receptor agonists (34,35), which also have the disadvantages of poor brain penetration and causing potent peripheral side effects (36).

In contrast to the well-defined potential of A₁ receptor activation to control seizures, the role of A_{2A} receptors is less well established. In fact, some reports with purportedly selective A_{2A} receptor agonists consistently observed anticonvulsant effects (32,37–39). However, despite the established presence of low amounts of A_{2A} receptors in cortical brain regions, it has recently been concluded that A_{2A} receptor agonists (like CGS 21680) mostly bind to A₁ rather than to A_{2A} receptors in cortical regions (8). This suggests that the activation of A₁ receptors might underlie the anticonvulsive effects of these purportedly selective A_{2A} receptor agonists (40). However, there are reasons to believe that A_{2A} receptors might play a role in the control of seizures and/or epileptogenesis. In fact, the group of Vaugeois showed that pharmacological blockade or genetic deletion of A_{2A} receptors decrease ethanol withdrawal-induced seizures in mice (10). Likewise, it has systematically been shown that blockade of A_{2A} receptors is neuroprotective in different noxious situations that involve limbic or cortical degeneration [reviewed in (9)], as is the case of temporal lobe epilepsy (41). This preliminary evidence, together with the presently observed robust increase in the density of adenosine A_{2A} receptors in the cerebral cortex of rats that had undergone a convulsive period, make A_{2A} receptor antagonists an attractive novel class of anticonvulsive drugs.

In conclusion, the presently observed long-term decrease in the density of A₁ receptors and the parallel increase of the density of A_{2A} receptors in the cerebral cortex provide a preliminary rationale for the development of novel anticonvulsive strategies targeting the adenosine neuromodulatory systems, which might be based on the combined use of strategies to burst the extracellular levels of adenosine to activate inhibitory A₁ receptors together with antagonists of facilitatory A_{2A} receptors.

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